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TITLE: Molecular Basis of Autophagy-Mediated Resistance to Radiation and Apo2L/TRAIL Therapy in Prostate Cancer Cells

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| 14. ABSTRACT Cell death and survival signaling pathways are important for the cellular response against ionizing radiation (IR) and chemotherapy in prostate cancer (PCa). IR and Apo2L/TRAIL, are widely used as therapeutics for PCa, however cellular resistance developed over the time may hinder their effectiveness. In this study we have investigated the response of Apo2L/TRAIL and IR in PCa cell lines. PC3 cells were more sensitive to Apo2L/TRAIL-mediated cell death as compared to LNCaP-derived C4-2. The cell death observed showed both apoptotic and non-apoptotic characteristics, suggesting that autophagy might also contribute to cytotoxicity. We therefore further investigated the role of autophagy in the IR and Apo2L/TRAIL response in PCa. Following Apo2L/TRAIL treatment of PC3 cells, microtubule-associated protein 1 light chain LC3, a classical marker for autophagy showed enhanced autophagosomal staining. In contrast, LC3 failed to associate with autophagosomes in C4-2 cells. Moreover, ATG7 levels were lower in C4-2 in comparison to PC3 cells. Furthermore, the ATG5-12 complex protein levels were decreased in PC3 cells while remaining unchanged in C4-2 cells. Sub-cellular fractionation revealed decreased ATG5 levels in the membrane fraction upon Apo2L/TRAIL and IR in PC3 cells. In C4-2 cells ATG5 was enriched in the soluble fraction upon IR while it decreased in both fractions upon Apo2L/TRAIL treatment. Real time PCR data showed differential expression of various autophagy related genes in PC3 and C4-2 cells. More cell death was observed in PC3 cells upon Apo2L/TRAIL treatment and was inhibited by inhibiting autophagy by 3-MA and/or by using pan-caspase inhibitor z-VAD, suggesting that autophagy induced by Apo2L/TRAIL results in caspase dependent cell death in PC3 cells. On the other hand, C4-2 cells did not show any cell death following Apo2L/TRAIL treatment, and inhibiting autophagy increased Apo2L/TRAIL-induced cell death in C4-2 cells. This indicated that autophagy leads to cell survival in C4-2 cells. These results suggest that autophagy plays opposite role in mediating cell death or survival in different PCa cell lines. Therefore, it is important to study the mechanism of autophagy mediated apoptosis or cell survival signaling to understand the different response towards Apo2L/TRAIL and IR therapy observed in different PCa patients. The status of autophagy signaling is important to decide the survival outcomes and use of pharmacological inhibitors can improve the efficacy of Apo2L/TRAIL and irradiation therapy in PCa. | | | | | |
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Title: Molecular basis of autophagy-mediated resistance to radiation and Apo2L/TRAIL therapy in prostate cancer cells

Introduction

Molecular signaling related to cell death and survival plays an important role in mediating the cellular response to radio- and chemotherapy in prostate cancer (PCa). Cell death can take place by various mechanisms that include programmed cell death Type I (apoptosis), programmed cell death type II, autophagy, and necrosis. Apoptosis involves intrinsic and extrinsic signaling followed by chromatin condensation and fragmentation, whereas autophagy mainly involves the bulk degradation of proteins and cellular organelles. Unlike apoptosis and autophagy, necrotic cell death involves plasma membrane breakdown and inflammation due to cytoplasmic spillage into the surrounding tissue (1). In this study I have investigated the role of autophagy signaling in PCa following treatment with ionizing radiation (IR) and Apo2 ligand (Apo2L)/TRAIL. Autophagy is broadly defined as a self degradation process, which can mediate both cell death as well as cell survival and has role in cellular differentiation and homeostasis. Proteins involved in autophagy are encoded as ATGs, with more than 20 such genes identified in yeast and human (2-3). Sequence of events including autophagy initiation, vesicle nucleation, retrieval/elongation of autophagosomes, docking/fusion with lysosomes and finally vesicle breakdown and degradation to complete autophagy signaling (4). Microtubule-associated protein 1 light chain 3 beta (LC3; also called ATG8) is a key autophagy regulatory gene required for autophagosome formation (5-7).

Upon autophagy induction, Pro-LC3 is cleaved by ATG4 and conjugated to phosphatidylethanolamine (PE) by ATG7 and ATG3 to form LC3 II which can then bind to internal membranes. Thus, detection of LC3 II is considered as the best marker for accumulation of autophagosomes and autophagy. Autophagy, which is induced during starvation, growth factor deprivation, hypoxia, endoplasmic reticulum (ER) stress, and microbial infection can prevent cell death (1). However, it can also induce cell death when there is excessive mitophagy that leads to loss of mitochondrial innermembrane potential, caspase activation, and lysosomal membrane permeabilization (LMP) followed by release of various cathepsins (lysosomal proteases) (8). Oncogenic activation of Ras, PI3K, Akt, and mTOR has been shown to inhibit autophagy, therefore Akt inhibitors facilitate cell death by inducing autophagy (9-10). On the other hand, the tumor suppressor p53 can have both promoting and inhibitory effect on autophagy depending upon the status of p53 that includes certain mutations and its subcellular distribution (10).

Autophagy has been shown to be a mechanism to overcome the cell death induced by various drug therapies in PCa. Autophagy is induced in LNCaP cells under androgen deprivation conditions and siRNA-mediated knockdown of Beclin1. Moreover use of the autophagy inhibitor 3-MA increased apoptosis in LNCaP cells suggesting a protective role of autophagy (11).

Apo2L/TRAIL (dulanermin) is used as a tumor-specific therapeutic that is currently tested in the clinic for various malignancies, including PCa (12). In a recent report, the Akt inhibitor nucleoside analog triciribine, has been shown to enhance Apo2L/TRAIL-mediated cell death in PC-3 and LNCaP cells (13).

Ionizing radiation (IR) is another commonly used therapy for PCa although often is not effective when used alone. Monoallelic loss of Beclin1, which is frequently observed in prostate tumors, and biallelic loss of ATG5, an important mediator of autophagy were shown to increase DNA damage induced foci, centrosome abnormalities, chromosome abnormalities and gene amplification (14-15). Therefore, autophagy has been reported to contribute to cell survival as well as cell death, depending upon cell context and the type of stimulus.

Results

Aim: To determine the role of autophagy in the resistance of PCa cells to Apo2L/TRAIL and IR treatment

a. Examine the protein and mRNA expression profile of autophagy regulating proteins

To investigate the change in mRNA expression of autophagy regulating proteins we performed real-time PCR in PCa cell lines PC3 and C4-2 cells at 6 hours following 10 Gy irradiation using Super Array readymade kit for autophagy genes (cat. No. PAHS 0084, SA Bioscience). The analysis was done using web-based software available at SA Bioscience for this array. The results obtained showed differential regulation of many autophagic regulatory genes within PC3 and C4-2 cells without any treatment (Fig 1A). Following IR there were many genes up regulated or down regulated as shown in the heat map for both PC3 and C4-2 cells (Fig 1B,C). ATG10, ATG4a, ATG4b, ATG4d, ATG9b, BECN1, PRKAA1 (AMPK), ULK1, ULK2, UVRAG, ATG3 and ATG16L2 were significantly down regulated in PC3 and up regulated in C4-2 cells (Fig 2A,B). ATG12, ATG5, ATG9a, HSP90aa1 and SQSTM1 were up regulated in PC3 and down regulated in C4-2 cells (Fig 2A,B). This suggested that genes required for autophagy induction such as ULK1, BECN1, ATG4a, and ATG4b, were up-regulated in C4-2 cells while genes required for autophagic clearance such as SQSTM1 were up-regulated in PC3 cells, indicating that there are differences in autophagic signaling at different steps in these two PCa cell line. Also UVRAG, known to be associated with UV resistance was up-regulated in C4-2 cells but down-regulated in PC3 cells, suggesting that UVRAG can also have a role in mediating resistance against radiation therapy. These mRNA changes need to be validated in terms of protein expression to derive a consensus for the role of autophagy in mediating cell death, cell survival and or developing resistance to radiation therapy. Several apoptotic genes that are known to be involved in autophagy regulation were also affected by irradiation in PCa cell lines. BAK1, BCL2, BCL2L1 and CASP8 were down-regulated in PC3 and up regulated in C4-2 cells (Fig 2C). These findings also need to be validated and explored in future to establish the interaction between autophagic and apoptotic signaling upon irradiation.

b. Determine the role of autophagy in cell death and survival

To investigate the effect of Apo2L/TRAIL and irradiation on cell death in PCa cell lines, cells were subjected to respective treatments and their sub-G1 DNA content was measured using flow cytometry. Cell death was induced in PC3 cells upon TRAIL treatment whereas C4-2 cells did not show any differences in cell viability, suggesting the existence of survival pathway (Fig 3A). Since autophagy can contribute to cell survival as well as cell death we then examined cell death in the presence of pharmacological inhibitors of autophagy. Autophagy signaling involves sequential steps consisting of formation of autophagosomes, followed by their fusion with lysosome and autophagic degradation of the cellular proteins and organelles. We utilized two inhibitors, 3-methyladenine (3-MA) and chloroquine, which act at different steps in autophagy signaling. 3-MA inhibits the induction of autophagy by inhibiting the activation of the PI3KclassIII kinase, while chloroquine inhibits autophagy degradation by inhibiting the acidification of autolysosomes. Therefore, depending upon the stage of autophagic signaling, both inhibitors can have a differential effect on cell death and survival mediated by autophagy. Combination of 3-MA with TRAIL resulted in reduction of cell death in PC3 cells suggesting that autophagy induction is required for TRAIL-induced cell death in PC3 cells (Fig. 3b). On the other hand 3-MA treatment with TRAIL sensitized C4-2 cells to TRAIL-induced cell death indicating that autophagy might serve as survival signaling in C4-2 cells (Fig 3B). Interestingly, addition of chloroquine slightly enhanced the effect of TRAIL in PC3 cells instead of rescuing the cell death suggesting that inhibition of autophagic degradation further enhanced cell death (Fig 3 c). In C4-2 cells addition of chloroquine lead to increased cell death upon TRAIL treatment, again confirming that inhibiting autophagic degradation can enhance cell death (Fig 3C). Addition of the caspase inhibitor z-

VADfmk inhibited cell death in PC3 with no effect on C4-2 cells suggesting that autophagy-mediated cell death in PC3 cells is caspase dependent (Fig 3D).

We next generated shRNA-mediated knockdowns specific for ATG7 and LAMP2, known to regulate autophagy induction and autophagy degradation steps in a very specific manner. To avoid the non specific effect of shRNA, we used as well as non-target shRNA and only vector shRNA controls with two different shRNA clones targeting two regions of the target mRNA for both genes. Cell death was observed in shATG7 and shLAMP2-expressing cells upon TRAIL treatment. In line with the previous data, shATG7 expressing PC3 cells showed a reduction in TRAIL-induced cell death while C4-2 cells with ATG7 knockdown were more sensitive to TRAIL treatment compared to the wild type, non-target and vector control cells (Fig 4A, B). LAMP2 knockdown showed a similar effect as did chloroquine, both enhancing the TRAIL-induced cell death in both PC3 and C4-2 cells (Fig 4C, D). Western blot analysis showed the extent of ATG7 and LAMP2 knockdown (Fig 4 E, F). Further cell death assay was performed in irradiated PC3 and C4-2 cells. PC3 cells showed cell death at 6 hours following irradiation which was decreased at 24 hours, with no effect observed in C4-2 cells (Fig 4G).

c. Examine the kinetics of autophagosomes formation

To study the kinetics of autophagosome formation we first examined the conversion of soluble LC3 I to the membrane bound form LC3 II, in PC3 and C4-2 cells upon TRAIL treatment. Levels of LC3 II were increased in PC3 compared to C4-2 cells, as shown by endogenous confocal immuno-staining for LC3 (Fig 5A). Soluble and membrane bound LC3 were examined in different fractions of cells obtained by ultra-centrifugation. LC3 II levels were increased in the membrane-bound fraction in PC3 compared to C4-2 cells (Fig 5B). To further test the turnover of LC3 II, we used chloroquine to inhibit the autophagic degradation and examined the levels of LC3 II. Upon chloroquine addition along with TRAIL, LC3 was accumulated in both PC3 and C4-2 cells (Fig 5C, D). A comparison between steady state and accumulated LC3 II levels suggested that LC3 turnover was faster in C4-2 compared to PC3 cells.

Autophagosome formation is a very dynamic process and requires recycling of the ATG5-12-16 protein complex. Once the autophagosome is completed the ATG5-12 complex falls off from the autophagosomal membrane. To examine the status of the membrane-bound ATG5-12-16 complex, we probed TRAIL-treated soluble and membrane-bound fractions with an antibody specific to ATG5, which recognizes the ATG5-12 conjugate and an antibody specific to ATG16L. ATG5-12 was found predominantly in the membrane-bound fractions of PC3. In contrast, it was found in the soluble fractions in C4-2 cells, suggesting a higher rate of recycling (Fig 6A, B).

Transmission electron micrographs confirmed the presence of more autophagic bodies in PC3 compared to C4-2 cells upon TRAIL treatment (Fig 7A). Chloroquine treatment with TRAIL resulted in higher accumulation of autophagic bodies in C4-2 cells suggesting enhanced autophagic degradation in C4-2 compared to PC3 cells (Fig 7B).

These findings were further supported by another assay used to determine the autophagis flux for LC3 by using the GFP-mCherry-LC3 fusion protein. During autophagy, LC3 II is recruited to the autophagosomal membranes and continues to be present on the membranes of completed autophagosomes, visualized as a yellow signal due to colocalization of GFP and mCherry. When autophagosomes fuse with lysosomes, because of the acidic pH, GFP fluorescence is diminished while mCherry still remains stable. Thus, by monitoring the conversion of yellow LC3 II puncta into only red LC3 II puncta, it enables to determine the rate of autophagosome conversion into autolysosomes allowing the autophagic flux to be measured. To perform this, we generated stable clones in PC3 and C4-2 cells expressing GFP-mCherry-LC3. Upon TRAIL treatment, we observed LC3 II puncta by confocal microscopy. PC3 cells showed only autophagosomal LC3 II as yellow puncta, whereas C4-2 showed

conversion of autophagosomal LC3 II (yellow) to autolysosomal LC3 II (red) within 3 hours of TRAIL treatment (Fig 8A). Addition of chloroquine accumulated autophagosomal LC3 II (yellow) in both cell lines (Fig 8B).

To further study levels of LC3 II conversion upon irradiation, we performed immunostaining for endogenous LC3 in irradiated (10 Gy) PC3 and C4-2 cells. LC3 II levels were higher in PC3 compared to C4-2 cells (Fig 9A). Moreover, ATG7 and ATG3, required for the conversion of LC3 II were higher in PC3 cells compared to C4-2 cells. Levels of ATG4a, required for recycling of LC3 II were higher in C4-2 compared to PC3 cells (Fig 9B). Localization of ATG5-12 and ATG16 showed increased basal level of membrane-bound puncta in C4-2 compared to PC3 cells, as shown by confocal immunostaining as well as cell fractionation, indicating a higher level of basal autophagy in C4-2 cells (Fig 10 A, B). Upon irradiation, the levels of ATG5-12 as well as ATG16 bound to membrane was decreased in both PC3 and C4-2 cells, suggesting that progression of autophagy leads to falling off the ATG5-12-16 complex from the autophagosomal membrane (Fig 10A, B).

KEY RESEARCH ACCOMPLISHMENTS:

- Irradiation leads to up-regulation of autophagy-mediating genes in C4-2 cells but not in PC3 cells.
- APO2L/TRAIL-induced cell death in PC3 cells was reduced upon inhibiting autophagy, indicating a prodeath function of autophagy.
- APO2L/TRAIL-induced autophagy and cell survival in C4-2 cells, which was diminished upon inhibiting autophagy, indicating that autophagy mediates cell survival in C4-2 cells.
- Irradiation did not induced cell death while addition of chloroquine sensitized PC3 and C4-2 cells to irradiation-induced cell death.
- LC3 turnover was faster in C4-2 compared to PC3 cells upon APO2L/TRAIL treatment.
- ATG5-12 was more membrane-bound in PC3 compared to C4-2 cells suggesting a higher rate of their recycling in C4-2 cells upon APO2L/TRAIL treatment.
- The autophagic flux was higher in C4-2 compared to PC3 cells upon APO2L/TRAIL treatment.
- Recruitment of LC3 and the ATG5-12-16 complex to autophagosomal membrane showed similar kinetics in PC3 and C4-2 following irradiation.

REPORTABLE OUTCOMES:

Singh K, Mazumder S, Almasan A. Presented abstract entitled: “Molecular basis of autophagy-mediated resistance to radiation and Apo2L/TRAIL therapy of prostate cancer”, AACR 101st Annual Meeting, 2010.

Almasan A, **Singh K**, Chatterjee P, Mazumder S. Presented abstract entitled: “A Cyclin E fragment enhances radiation-induced cell death by modulating autophagy”, AACR 101st Annual Meeting, 2010.

Singh K, Mazumder S, Almasan A, Presented abstract entitled as “Molecular basis of autophagy-mediated resistance to radiation and Apo2L/TRAIL therapy of prostate cancer”, CSHL, meeting on “Mechanisms & Models of Cancer”, August, 2010.

CONCLUSION:

In this study I have investigated the role of autophagy in mediating cell death and cell survival in PCa cell lines. I have used Apo2L/TRAIL and IR, two major therapeutics widely used for PCa treatment, to study the cell death signaling pathway. It was observed that both Apo2L/TRAIL and IR can induce autophagy in PC3 and C4-2 PCa cell line, but the signaling events were different in PC3 and C4-2 cells and henceforth the effect on cell survival. Upon Apo2L/TRAIL treatment, PC3 exhibit autophagy induction but no clearance of autophagosomes leading to initiation of apoptosis and cell death. Inhibiting autophagy induction reduced the Apo2L/TRAIL-

induced cell death indicating that the incomplete autophagy observed in PC3 cells can lead to cell death. In C4-2 cells, Apo2L/TRAIL induces autophagy that reaches the completion as evident by LC3 II localization in autolysosomal compartment and degradation. This was responsible for a better cell survival of C4-2 cells following Apo2L/TRAIL treatment, suggesting that complete autophagy can provide as cell survival signaling. Importantly, inhibiting autophagy in C4-2 cells resulted in increased Apo2L/TRAIL-induced cell death. Therefore, the response to Apo2L/TRAIL can be predicted based on the status of autophagy signaling. Unlike Apo2L/TRAIL, IR did not induce cell death in PC3 and C4-2 cells, but induced autophagy in both the cell line. Future studies are required to evaluate the role of autophagy in cell survival following IR. mRNA profiling of various autophagy genes suggested basal differences in expression of autophagy-related genes in PC3 and C4-2, therefore, may not be sufficient to conclude the cell survival outcomes. Thus, investigating the molecular modifications of autophagy-related proteins will need to be pursued. This will also help to identify diagnostic marker related to autophagy in PCa.

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APPENDICES:

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
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| INSTITUTION AND LOCATION | DEGREE (if applicable) | YEAR(s) | FIELD OF STUDY |
| B. R. Ambedkar University, Agra, India | B.Sc. | 1999 | Botany, Zoology, Chemistry |
| B. R. Ambedkar University, Agra, India | M.Sc. | 2001 | Biochemistry |
| Anna University, Chennai, India | M.Tech. | 2003 | Biotechnology |
| National Center for Cell Science, Pune University, Pune, India | Ph.D. | 2008 | Biotechnology |

A. Personal Statement

Cancer development is a consequence of a succession of a series of complex cellular events that can be broadly classified as gene alterations, protein structural and functional defects, and dysfunctional cellular signaling events. Increased mutations and genomic instability due to various cellular stresses leads to predisposition to tumor initiating and promoting conditions. My research has focused on studying the molecular signaling events that can play a key role in tumorigenesis and tumor suppression. Earlier I have worked on characterization of a chromatin remodeling tumor suppressor protein SMAR1 in breast cancer and its crosstalk with p53, NF- κ B, and Bax regulation pertinent to cell cycle and apoptosis. Later I have worked on the role of Metastasis tumor antigen 1, MTA1 in modulation of TGF β signaling and breast cancer metastasis.

My recent research interest has been to investigate cell death signaling pathways, apoptosis and autophagy in cancers of epithelial origin, such as prostate and lung cancer. Autophagy is induced in PCa following Apo2L/TRAIL and irradiation and mediates differential signaling events that decide the cell fate towards survival or apoptotic/autophagic cell death in different cell types. Incomplete autophagy in PC3 PCa cells leads to accumulation of autophagosomes and polyubiquitinated protein aggregates that triggers Caspase-8 cleavage and cell death. In contrast a complete and faster autophagy that is observed in LNCaP derived C4-2 cells facilitates cell survival. Inhibition of autophagic signaling inhibited cell death in PC3 cells whereas it induced cell death in C4-2 cells, indicating a dual role of autophagy in mediating cell survival and cell death outcomes depending upon cell type. In another related study I am in the process of characterizing the role of a proteolytic fragment of Cyclin E (p18-Cyclin E) in radiation-induced autophagy and senescence that consequently affect the cell survival in H1299 lung cancer cells. We have found that cells expressing p18-Cyclin E undergo apoptosis as an early response against irradiation. However, once p18-Cyclin E is stably expressed, a higher rate of autophagic signaling ensues to sustain their survival against an apoptotic signal. Additional studies revealed that autophagy can lead to senescence, which affects long-term cell survival as determined by colony formation assay. Inhibition of autophagic signaling reduced the development of the senescent phenotype. I am also involved in other related ongoing projects in the laboratory where we are interested to explore the role of p18-Cyclin E in DNA damage and repair signaling, and regulation of autophagy upon treatment with DNA damaging drugs of hematopoietic tumor cells.

Positions and Honors.

06/2002-12/2002: Tata Institute for Fundamental Research, Mumbai, Maharashtra, India

02/2003-06/2007: National Center for Cell Science, Pune, Maharashtra, India

06/2007-09/2008: Research Intern at M. D. Anderson Cancer Center, Houston, Texas, USA

10/2008- Present: Post Doctoral Fellow at Lerner Research Institute, Cleveland, Ohio, USA

Honors and Awards:

Award for Lectureship from Council of Scientific and Industrial Research (CSIR), Government of India in the year of 2001.

Junior and Senior Research Fellowship for two years, each from the Indian Council of Medical Research (ICMR), Government of India, in the year of 2001.

University Gold medal award in M.Tech. (Biotechnology) at Anna University, Chennai in the year of 2003.

Award for First Best Poster at “International Symposium on Translational research: Apoptosis and Cancer-2005”

Awarded for two years DOD training fellowship in Prostate Cancer Research Program, for the project entitled: “Molecular basis of autophagy-mediated resistance to radiation and Apo2L/TRAIL therapy in prostate cancer cells”, 05-2010 to 04-2012.

The F. Merlin Bumpus Junior Investigator Award semifinalist, for the project entitled as “A p18-Cyclin-E fragment enhances radiation-induced cell death by modulating autophagy”, September, 2010.

Selected for the CSHL workshop entitled “*Immunocytochemistry, in situ hybridization, super-resolution & live cell imaging*”, October, 2010.

A. Selected publications (in reverse chronological order):

Singh K, Matsuyama S, Almasan A. Ionizing radiation-induced autophagy and senescence is enhanced by p18-Cyclin E expression and results in reduced cell survival. (Manuscript communicated in Cell Death and Differentiation; partially presented at 101st Annual AACR Meeting, 2010)

Singh K, Almasan A. Apo2L/TRAIL mediated autophagic response confers cell death and survival outcomes in prostate cancer. (Manuscript in preparation; partially presented at 101st Annual AACR Meeting, 2010 and CSHL meeting on “Mechanisms & Models of Cancer”, 2010).

Singh K, Almasan A. Irradiation induced autophagic response in prostate cancer. (Manuscript in preparation; partially presented at 101st Annual AACR Meeting, 2010 and CSHL meeting on “Mechanisms & Models of Cancer”, 2010).

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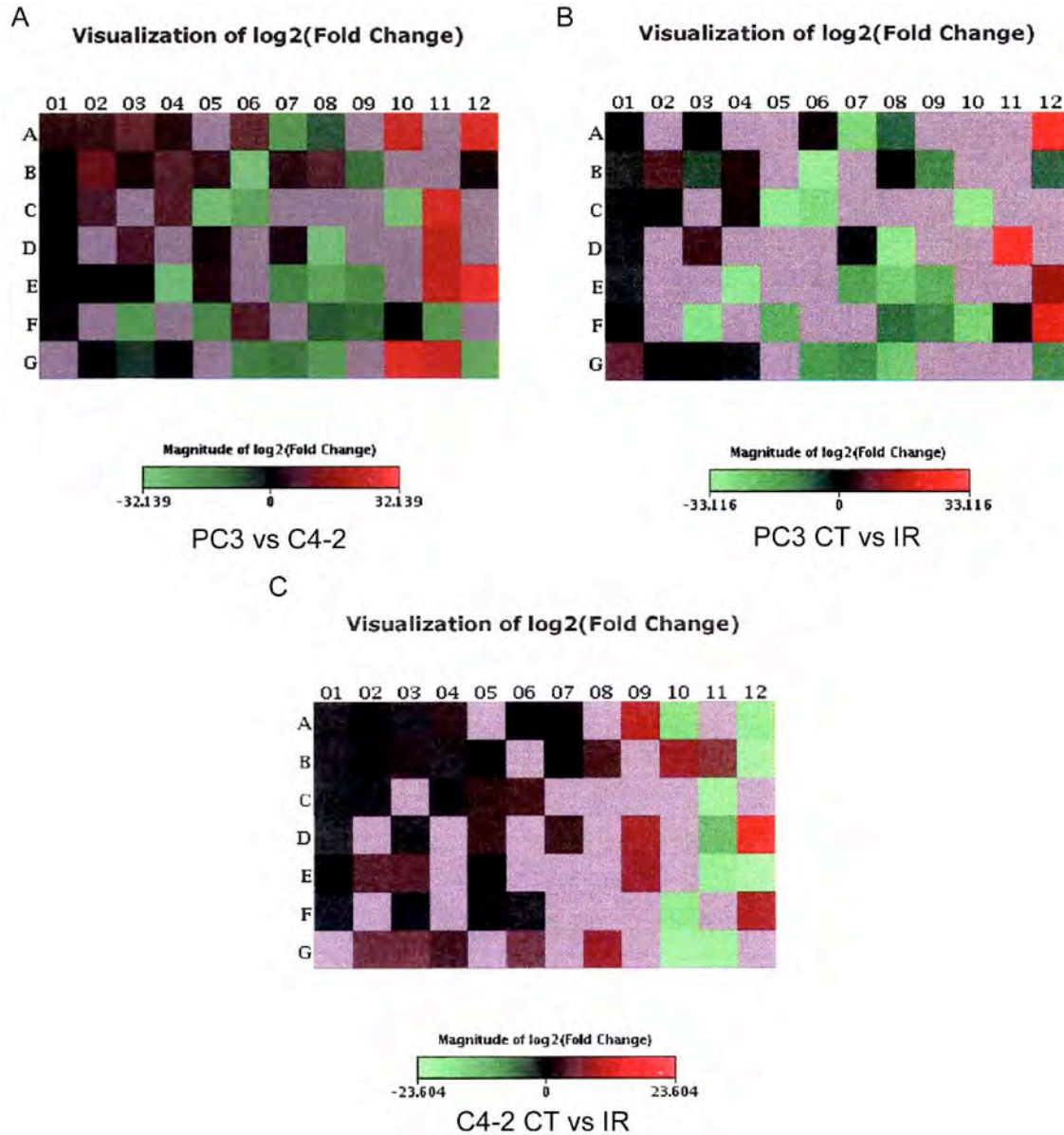
Published Abstracts (Posters Presented at National Conferences):

Singh K, Mazumder S, Almasan A. Presented abstract entitled: “Molecular basis of autophagy-mediated resistance to radiation and Apo2L/TRAIL therapy of prostate cancer”, AACR 101st Annual Meeting, 2010.

Almasan A, **Singh K**, Chatterjee P, Mazumder S. Presented abstract entitled: “A Cyclin E fragment enhances radiation-induced cell death by modulating autophagy”, AACR 101st Annual Meeting, 2010.

Singh K, Mazumder S, Almasan A, Presented abstract entitled as “Molecular basis of autophagy-mediated resistance to radiation and Apo2L/TRAIL therapy of prostate cancer”, CSHL, meeting on “Mechanisms & Models of Cancer”, August, 2010.

SUPPORTING DATA:



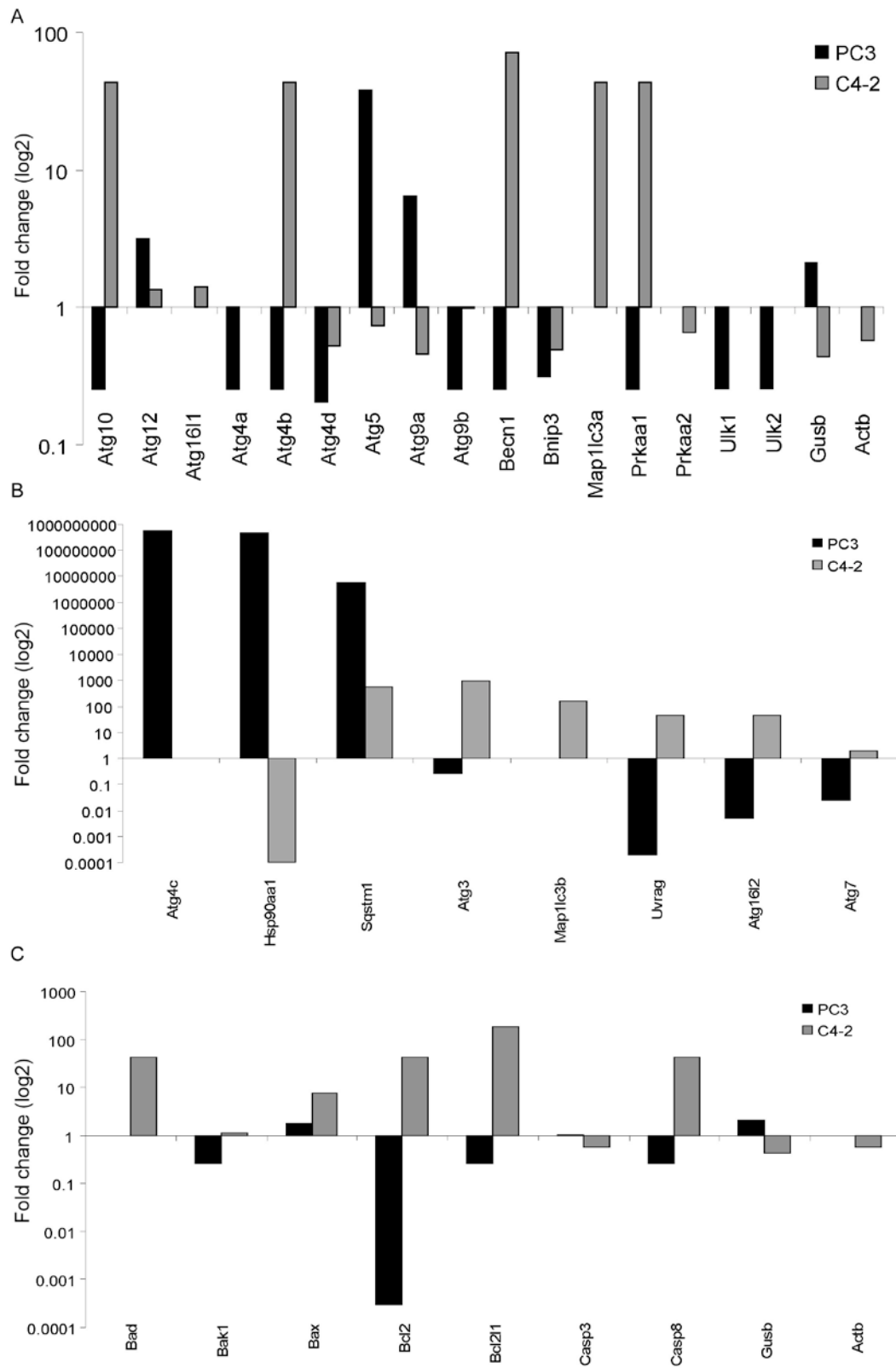


Figure 2. Real time PCR analysis for genes involved in autophagy signaling in PCa cells PC3 and C4-2 at 6 hours following 10 Gy irradiation. Fold change was determined relative to untreated control sample. Gusb and Actb served as housekeeping controls.

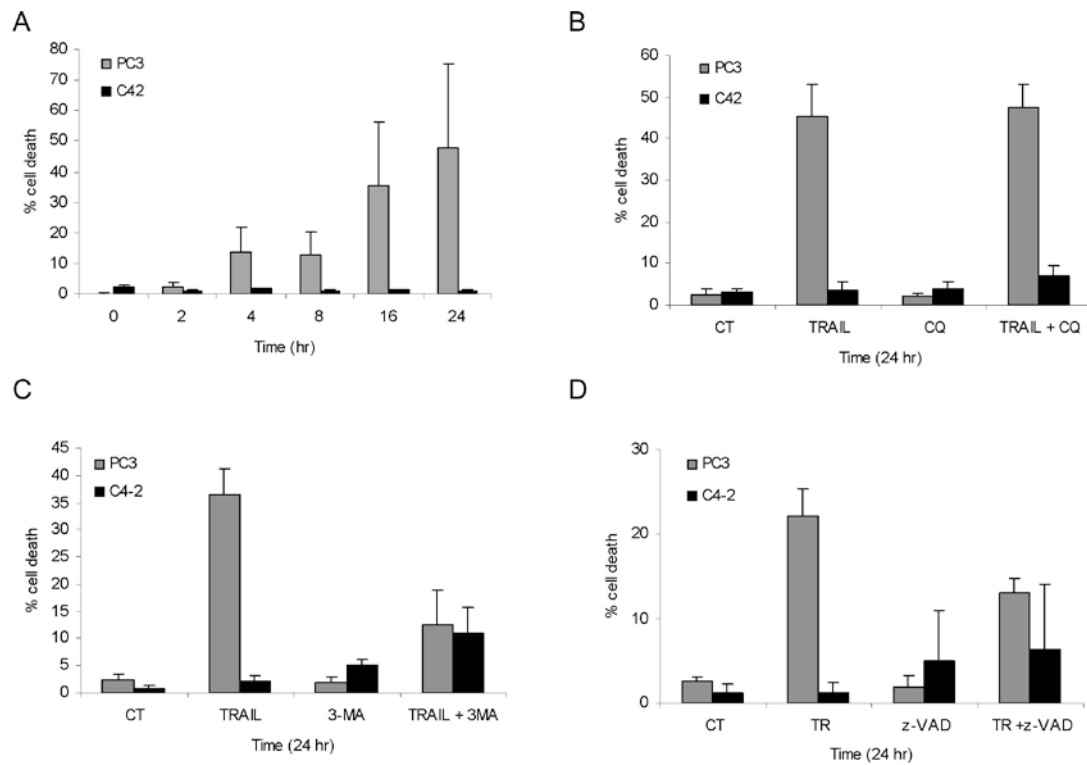


Figure 3. Apo2L/TRAIL-induced autophagy lead to cell death in PC3 cells, while enhanced cell survival in C4-2 cells. (A) Cell death is shown as percentage of cells with sub-G1 DNA content in PC3 and C4-2 cells treated with Apo2L/TRAIL (100nM) for the indicated time. (B, C) Cell death is shown as percentage of cells with sub-G1 DNA content in Apo2L/TRAIL-treated for 24 hours with or without Chloroquine (CQ; 100 μ M) or 3-Methyladenine (3-MA; 10 mM) in PC3 and C4-2 cells. (D) Cell death is shown as percentage of cells with sub-G1 DNA content in Apo2L/TRAIL (100 μ M) treated for 24 hours in the absence or presence of z-VAD (20 μ M) in PC3 and C4-2 cells.

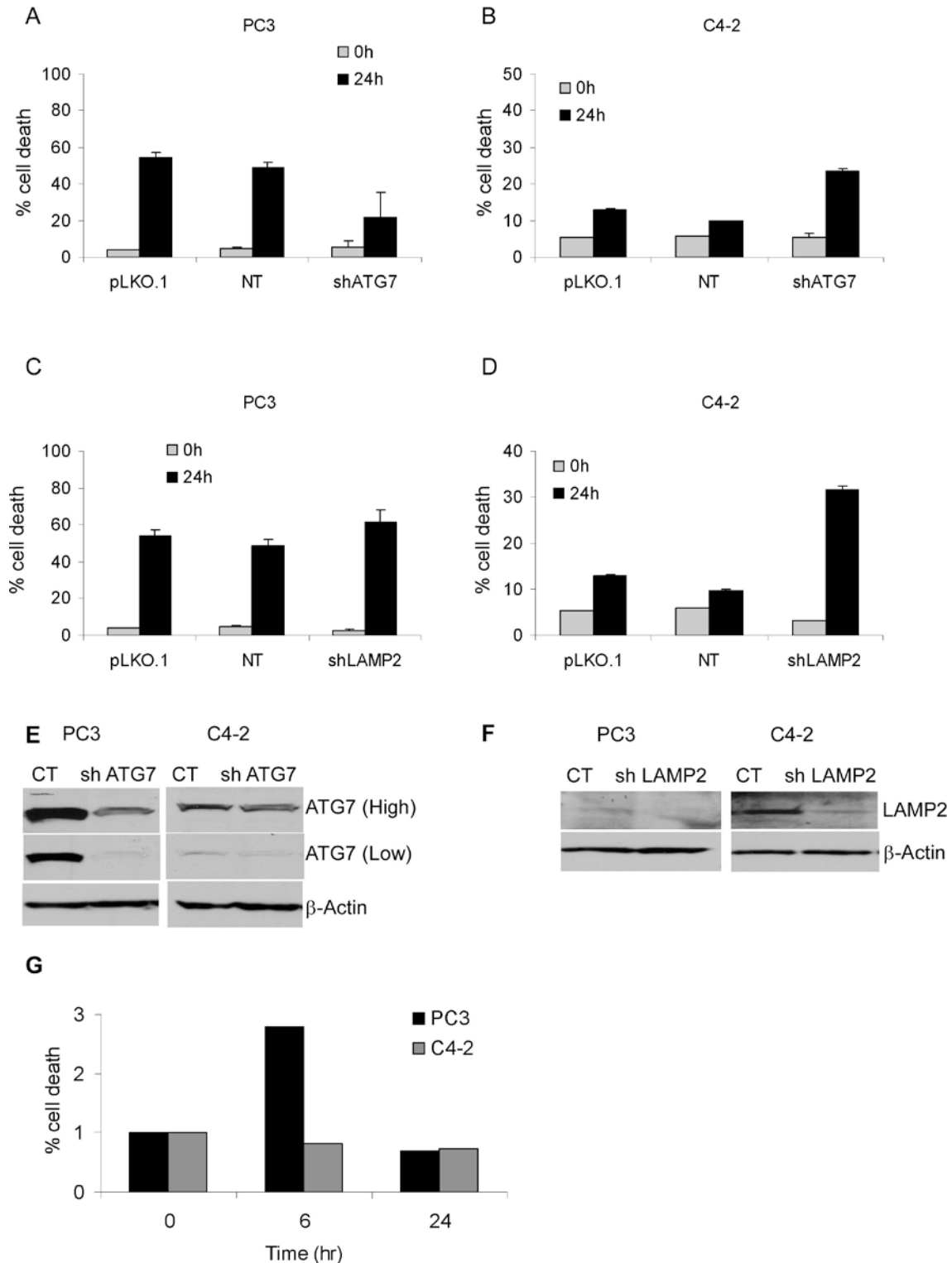


Figure 4. Autophagy inhibition decrease cell death in PC3, while it sensitized C4-2 to Apo2L/TRAIL- induced cell death. (A, B, C, and D) Cell death is shown as percentage of cells with sub-G1 DNA content in Apo2L/TRAIL(100nM) treated PC3 and C4-2 cells with or without shATG7 or shLAMP2 expression after 24 hours of treatment.(E,F)

Western blot analysis for ATG7 and LAMP2 in shATG7 or shLAMP2-expressing PC3 and C4-2 cells. (G) Cell death is shown as percentage of cells with sub-G1 DNA content in PC3 and C4-2 cells following irradiation (10 Gy).

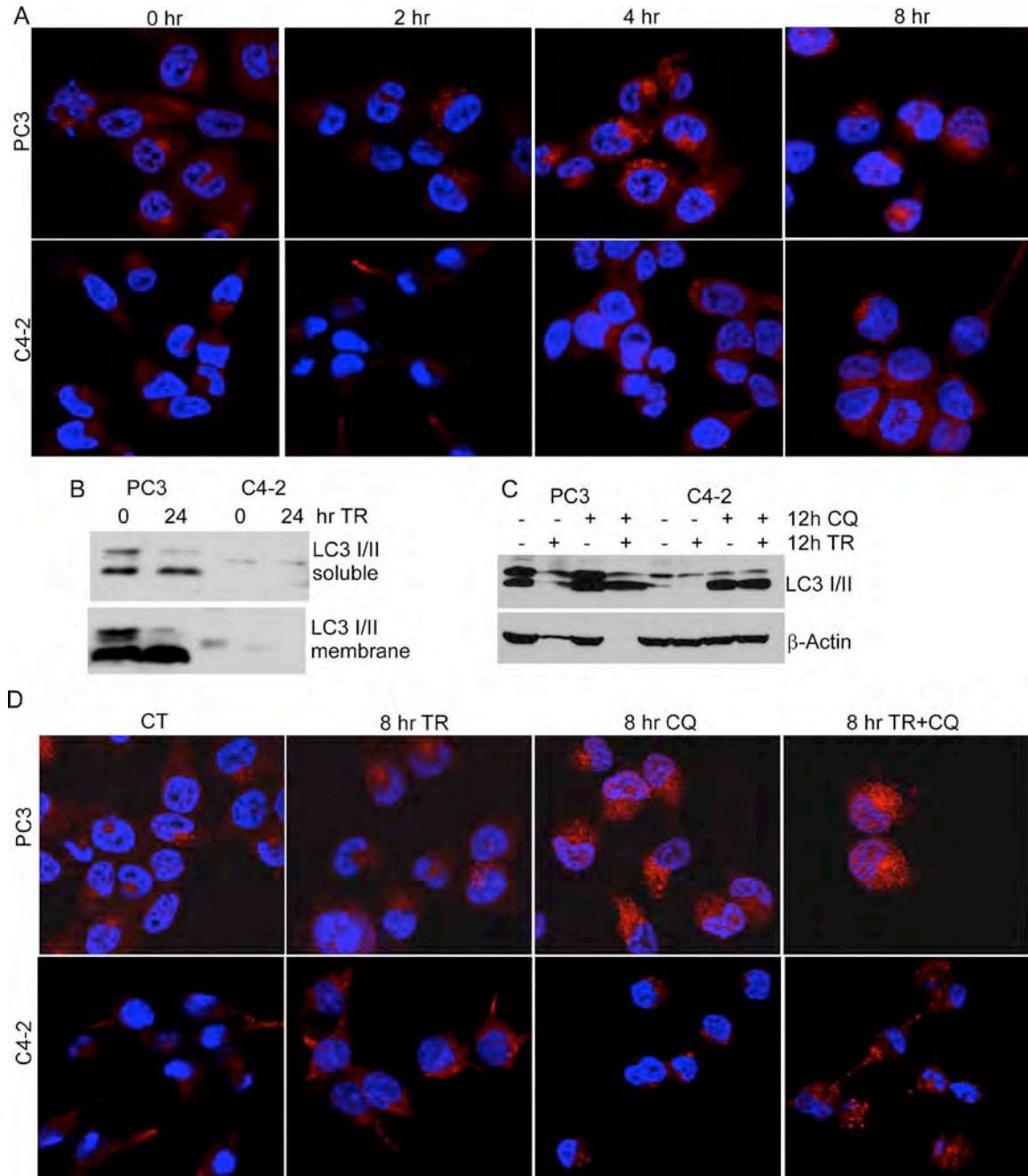


Figure 5. Apo2L/TRAIL-induced autophagy in PCa cell lines. (A) Confocal immunostaining for LC3 I/II in PC3 and C4-2 cells upon Apo2L/TRAIL (100nM) treatment for the indicated time. (B) Soluble and membrane-bound fractions from PC3 and C4-2 cells were obtained by ultra centrifugation and probed with anti-LC3 to detect LC3 I/II following Apo2L/TRAIL (100nM) treatment for 24 hours. (C) Cells were lysed at the indicated time following Apo2L/TRAIL (100nM) treatment with or without

chloroquine (100 mM) and immunoblotted for LC3 I/II. β -actin served as loading control. (D) Visualization of endogenous LC3I/II at 24 h following Apo2L/TRAIL (100nM) treatment in the absence or presence of chloroquine (100 mM). Nuclei were stained with DAPI.

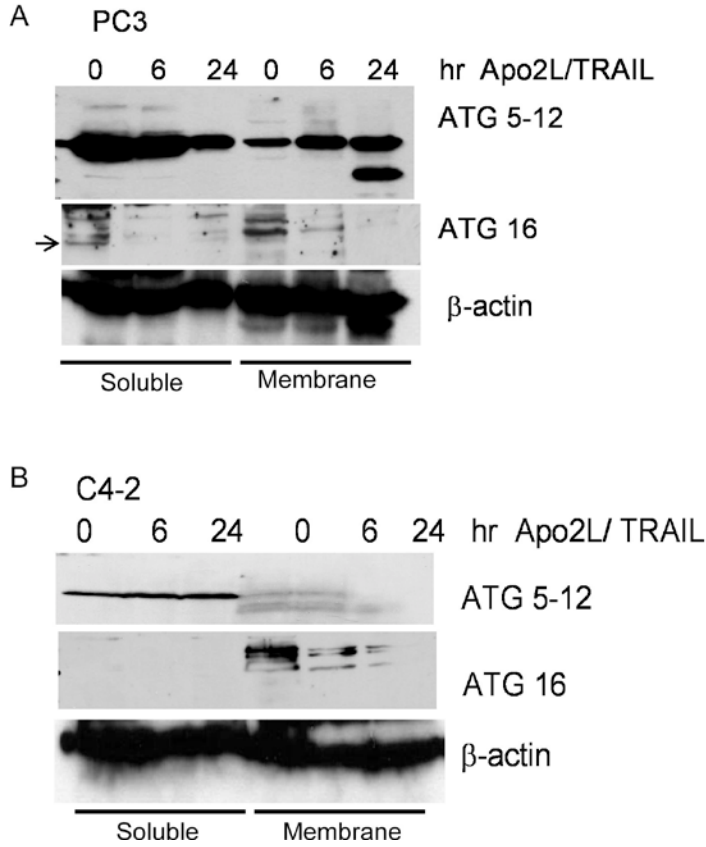


Figure 6. (A and B) Soluble and membrane-bound fractions from PC3 and C4-2 cells were obtained by ultra centrifugation and probed with anti-ATG5 to detect ATG5-12 conjugate and anti-ATG16 following Apo2L/TRAIL (100nM) treatment for 24 hours. β -actin served as loading control.

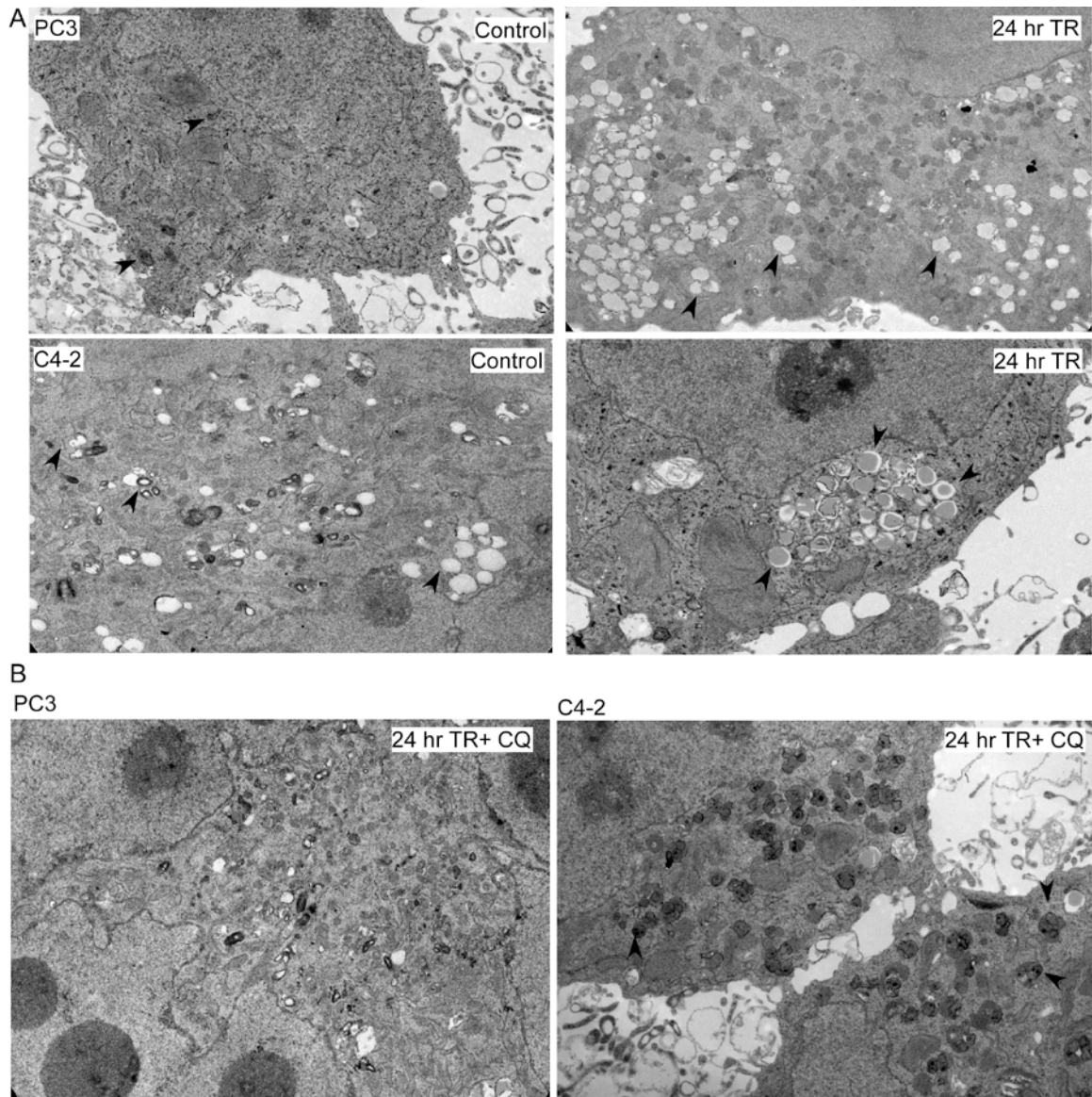


Figure 7. Increased accumulation of autophagic structures in C4-2 cells compared to PC3 upon Apo2L/TRAIL and chloroquine treatment (A,B) Transmission electron microscopic analysis of autophagic structures at 24 h following Apo2L/TRAIL (100 nM) treatment with or without chloroquine (100 μ M).

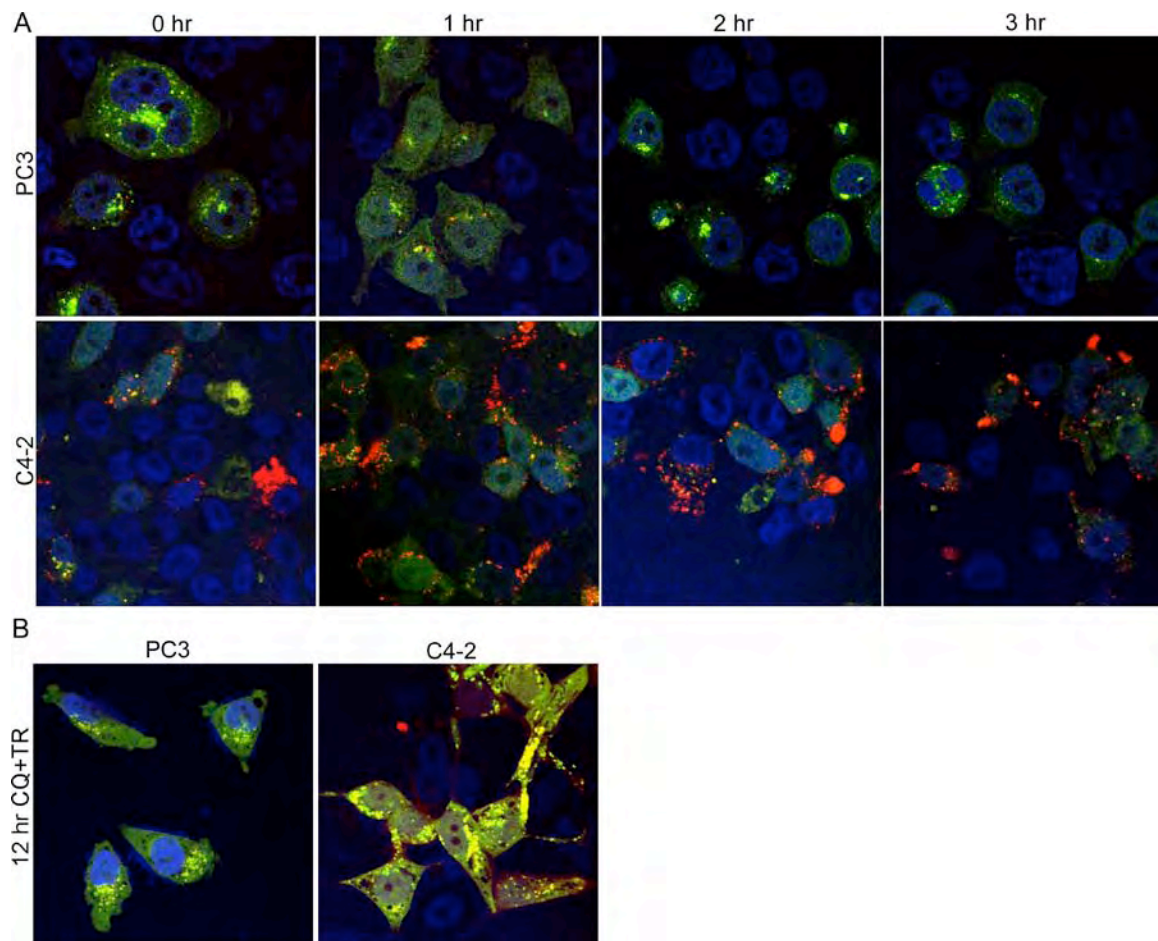


Figure 8. Autophagic flux is higher in C4-2 cells compared to PC3 cells. (A and B) Autophagy flux shown by confocal microscopic images for cells stably-expressing GFP-mCherry-LC3 in PC3 and C4-2 cells following Apo2L/TRAIL (100 nM) treatment with or without chloroquine (100 μ M).

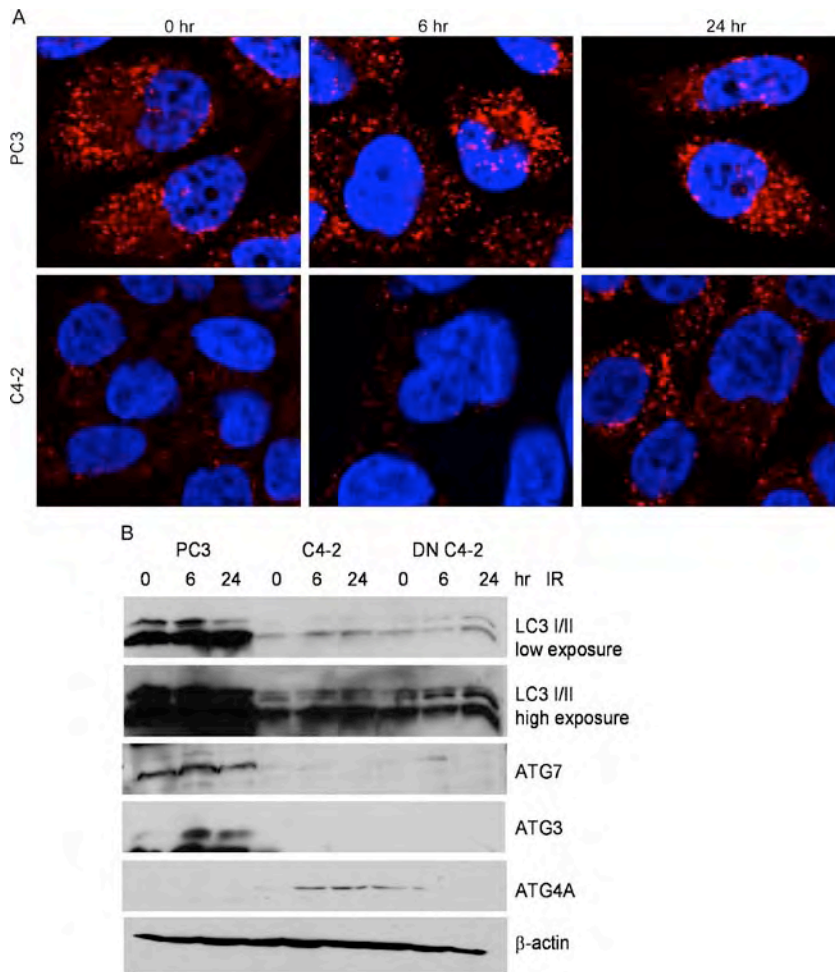


Figure 9. (A) Confocal immunostaining for LC3 I/II in PC3 and C4-2 cells following irradiation (10 Gy) for the indicated time. (B) Total lysate from PC3, C4-2, and DN-C4-2 (p53 mutant) cells following irradiation (10 Gy) were probed with anti-LC3, ATG7, ATG3, and ATG4a. β -actin served as loading control.

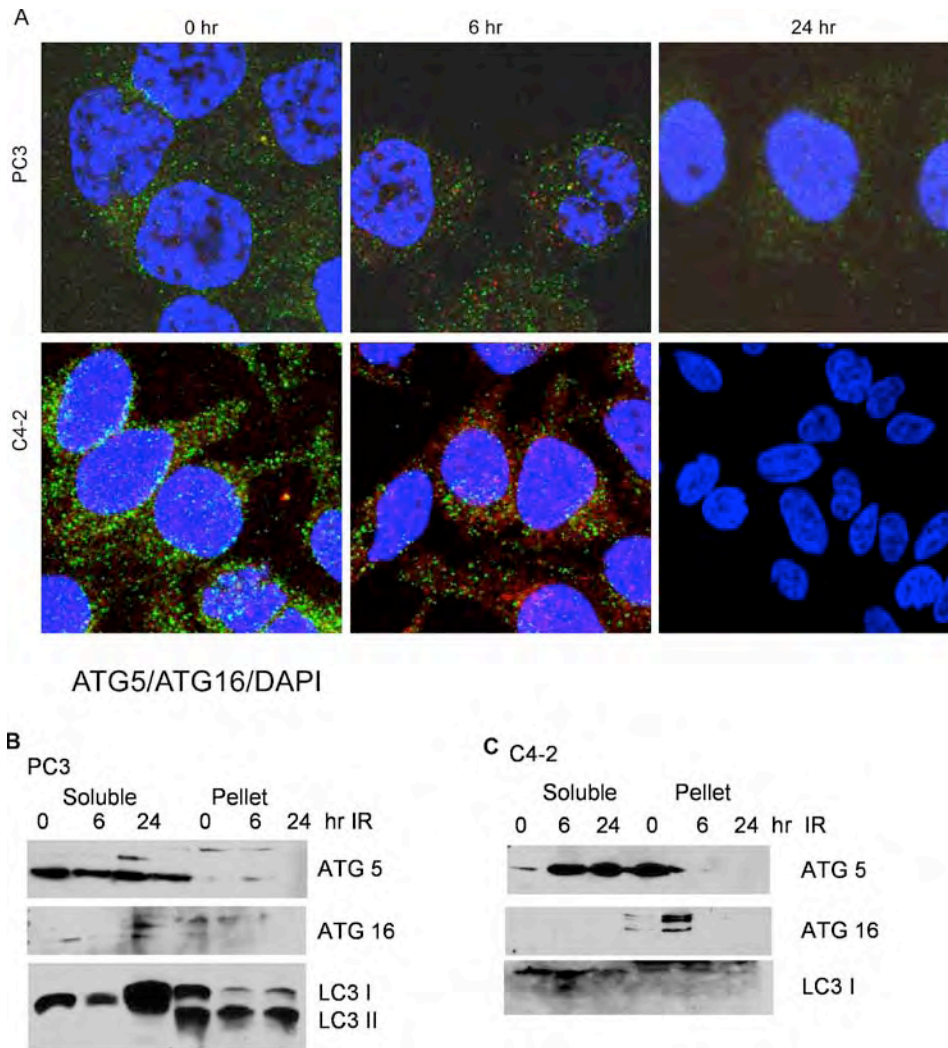


Figure 10. (A) Confocal immunostaining for ATG5 (green) and ATG16 (red) in PC3 and C4-2 cells following irradiation (10 Gy) for the indicated time. (B) Soluble and membrane bound (pellet) fractions from PC3 and C4-2 cells were obtained by ultra centrifugation and probed with anti-LC3, anti-ATG5 to detect ATG5-12 conjugate and anti-ATG16 at 24 hours following irradiation (10 Gy).